Eur. J. Biochem. 38, 497–506 (1973)

Enzymatic Iodination of Protein

Kinetics of Iodine Formation and Protein Iodination Catalyzed by Horse-Radish Peroxidase

Jacques POMMIER, Louis SOKOLOFF, and Jacques NUNEZ

Unité de Recherche sur la Glande Thyroïde et la Régulation Hormonale de l'Institut National de la Santé et de la Recherche Médicale, Bicêtre, and Section on Developmental Neurochemistry, Laboratory of Cerebral Metabolism, National Institute of Mental Health, Bethesda, Maryland

(Received April 4, 1973)

1. The initial rate of I_2 formation catalyzed by horse-radish peroxidase exhibited a clear sigmoid relationship with respect to the concentration of I^-. These data were found to fit an equation based on a model which predicts a second-order dependence on iodide concentration and are consistent with a bimolecular reaction between two I^- ions on the surface of the enzyme. Such a model presumes two sites for the substrate on the enzyme.

2. The initial rate of lysozyme iodination also exhibited a clear sigmoid relationship with respect to concentration of I^-. The sigmoidicity increased with the lysozyme concentration. These experimental data fit a random-ordered sequence of substrate fixation but with one of the two possible sequences kinetically preferred. These results also suggest that lysozyme interacts with the enzyme and that there are two sites for substrate addition on the surface of the enzyme.

3. When the ratio of the concentrations of both substrates, iodide and lysozyme, is varied, the rates of formation of each product, I_2 or iodinated protein, also vary; the lower the iodide/protein ratio, the lower the I_2 yield and the higher the rate of protein iodination. Studies on the influence of pH on the nature of the product showed that I_2 formation is favored at acidic pH and protein iodination at more alkaline pH. These results are also consistent with a two-site model for the enzyme, both sites being able to fix either two iodide ions or one iodide ion and one lysozyme molecule. The affinity of the sites for each one of the two substrates differs according to the pH. This conclusion was confirmed by the observation that iodination of free tyrosine could be obtained with very good yields provided that the pH of the reaction was adjusted to avoid either complete dimerization of free tyrosine, which is favored at alkaline pH, or I_2 formation which is favored at low pH.

4. These results and other quantitative data on the stoichiometry of H_2O_2 consumption do not establish unequivocally which is the oxidized iodide-reacting species, I^+ or I°. However, they indicate that I_2 is not the iodinating species in the protein iodination reaction catalyzed by horse-radish peroxidase.

Previous studies [1,2] have suggested that the chemical mechanism of the horse-radish-peroxidase-catalyzed iodination of protein is of a radical addition type and that the two radical intermediates. I° and the protein radical, are fixed on the enzyme, presumably in an intermediate complex. The present study provides additional evidence for two substrate sites, on the basis of the kinetics of iodide oxidation and I_2 formation and of protein iodination. These two sites, however, are not exclusively specific for each of the two substrates. These data and certain kinetic properties of the protein-iodination reaction, particularly the sigmoid relationship between initial reaction velocity and iodide concentration, have led us to consider further the question of the order of the fixation of the two substrate to their respective sites and the sequence of the formation of the two radicals. The kinetics of the protein-iodination reaction have been found to be consistent with a random-ordered sequence of substrate fixation and radical formation, but with one of the two possible sequences kinetically preferred. This kinetic model is fully capable of explaining the sigmoid nature of the velocity-substrate concentration relationship. Furthermore, studies on the influences of pH on the nature of the product formed, either I_2 or iodinated protein, support by another experimental approach, the two-site model.

Enzymes. Horse-radish peroxidase (EC 1.11.1.7); glucose oxidase (EC 1.1.3.4).

MATERIALS AND METHODS

Products

Horse-radish peroxidase (RZ = 3) and glucose oxidase (grade A) were purchased from Boehringer, lysozyme from Calbiochem, hydrogen peroxide from Merck. All other products and chemicals were of the highest purity available from Prolabo. ^{125}I was purchased from the Commissariat à l'Energie Atomique.

I_2 Formation from I^-

Horse-radish peroxidase catalyzes the oxidation of I^- to form I_2, which in the presence of excess I^- forms I_3^-. I_3^- formation can be assayed by its absorbance at 353 nm [3]. Oxidation of I^- was followed by this method in a Cary-15 spectrophotometer. The experimental conditions of the assays were: 2 μg glucose oxidase, 8 mM glucose, 10—32.5 nM horse-radish peroxidase depending on the pH used for the reaction and 0.5—8 mM KI. The assays were carried out in a final volume of 1 ml at 23 °C. Under these conditions the time course of I_3^- formation was linear; the values thus obtained for the initial velocity were corrected for the equilibrium constant, K_d, of the reaction, $I + I_2 \leftrightarrows I_3^-$, as follows: $v = \Delta A/\text{min} \times [(K_d + c)/c]$ where c = iodide concentration. The value for K_d was established as 1.35 mM, which is very similar to that found by others [3,4].

Protein and Free Tyrosine Iodination

The method used for the study of the kinetics of protein iodination has been previously described [2]. Free tyrosine and monoiodotyrosine iodination was measured by the same technique.

Determination of the Optimal H_2O_2 Concentration

It is well known that H_2O_2, when in excess, is inhibitory [5—7]. The optimal level of H_2O_2 varies with the substrate concentration [5]. On the other hand, the kinetics of I_2 formation and protein iodination must be studied with non-limiting amounts of H_2O_2. For these reasons, the optimal amount of H_2O_2 was determined for each iodide concentration used. Separate assays were performed with a fixed, saturating concentration of glucose but with varying concentrations of glucose oxidase. The steady-state level of H_2O_2 thus produced was established for each I^- concentration. The amount of glucose oxidase finally chosen for the kinetic studies was one which was neither inhibitory nor limiting for all the iodide concentrations used with this glucose oxidase concentration. The proportionality between the initial rate of I_2 formation and the peroxidase concentration was also maintained.

Stoichiometry of H_2O_2 Utilization at High Enzyme Concentration

Some experiments on I_3^- formation and protein iodination were performed under the conditions described by Roman and Dunford [8]. Hydrogen peroxide (10 μM) was added to a 1-ml cuvette containing peroxidase (4.4 μM) and iodide (0.25 mM or 10 mM). The experiment was performed at two pH levels, 7.24 and 8.4 (0.05 M phosphate buffer), *i.e.* under the conditions used by Roman and Dunford [8]. I_2 formation was measured with a Cary-15 spectrophotometer, 30 s after the addition of H_2O_2. To measure peroxidase iodination, iodide was labeled with ^{125}I and the amount of iodinated enzyme was determined by the technique [2] used for lysozyme.

RESULTS

Kinetics of Iodide Oxidation and I_2 Formation

The rate of the oxidation of I^- to I_2 by horse-radish peroxidase was studied under conditions in which the reaction was linear with time, and H_2O_2 was generated at a rate sufficient to maintain all free enzyme in the compound I state, assuming, of course, that the reaction between the peroxidase and the H_2O_2 is not rate limiting [5]. Under these conditions it was found that the initial rate of I_2 formation exhibited a clear sigmoid relationship with respect to the concentration of I^- (Fig. 1A). Curve-fitting by means of the MLAB program for the PDP-10 computer [9] clearly indicated that the data in Fig. 1A were fitted far better by a sigmoid curve described by an equation of the type

$$v = \frac{k_1 [I^-]^2}{[I^-]^2 + k_2 [I^-] + k_3}$$

than by a straight line or a parabolic curve characteristic of a typical Michaelis-Menten relationship. This equation predicts that the initial velocity would be second order with respect to iodide concentration at low concentrations of I^- and would progress with increasing iodide concentration through a first order to eventually a zero-order relationship. These kinetics are consistent with a bimolecular reaction between two ions of I^- on the surface of the enzyme and suggest, therefore, the existence of a ternary complex of one enzyme molecule with two iodide ions (Fig. 2). This mechanism contrasts with an alternative mechanism by which one iodide ion at a time is oxidized and released as a free radical with two $I^\cdot$ atoms or one I^+ and one I^- ion in the medium then forming I_2 non-enzymatically. These latter mechanisms would not yield second-order kinetics nor a sigmoid relationship between initial velocity and iodide concentration; they are, therefore, excluded as a major mechanism of I_2 formation, at least under the usual

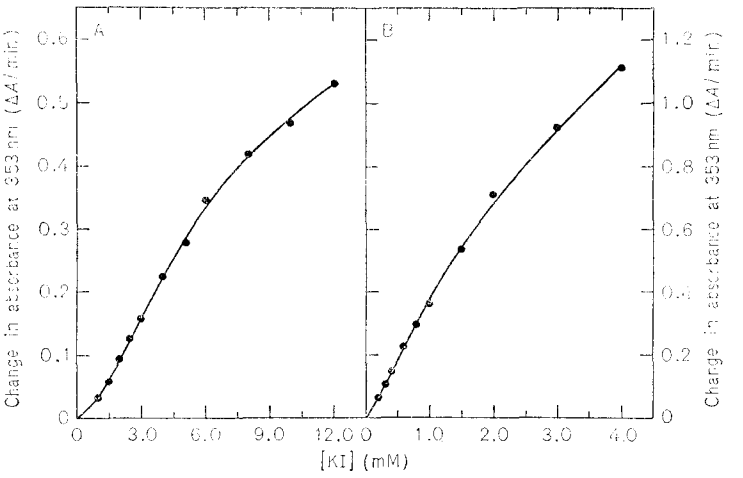

Fig. 1. *Influence of iodide concentration on the initial rate of horse-radish-peroxidase-catalyzed oxidation of iodide to iodine.* The reaction mixtures in (A) contained 0.05 M phosphate buffer pH 6.9, 5 mM glucose, 33 nM glucose oxidase and 25 nM peroxidase. The composition of the reaction mixtures in (B) were similar, except that the glucose oxidase concentration was 20 nM and the pH of the buffer was 5.3. The iodide concentrations were varied as indicated in the figures. The reactions were carried out at 24 °C. Each point represents the mean of the experimentally determined values in five experiments. The solid line is the theoretically computed best fitting curve of all the data to the equation, $v = k_1[I^-]^2/([I^-]^2 + k_2[I^-] + k_3)$, the form of the equation derived on the basis of a two-site model. (●) Experimental data and ( ) theoretical fit to two-site model

$$E_I + A \rightleftharpoons EA + A \rightleftharpoons EAA \rightleftharpoons E + AA$$

Fig. 2. *Reaction scheme for 2-site model of peroxidase-catalyzed oxidation of iodide to iodine showing ternary complex between 1 mole peroxidase · H_2O_2 and 2 moles iodide.* E_I represents peroxidase · H_2O_2 complex (complex I) and A represents iodide ion or iodine atom

conditions of our assay. It is possible that under some circumstances one or the other is operative since the degree of sigmoidicity observed in our studies did vary with the pH. For example, sigmoidicity was marked at pH 6.9 but considerably reduced at pH 5.3 (Fig. 1 B).

Kinetics of Lysozyme Iodination

We have previously noted that the process of iodination of a protein, such as lysozyme, exhibits features consistent with two sites of the enzyme [2]. For example, the protein eventually becomes inhibitory as its concentration is raised, but this inhibitory effect is antagonized by increasing the iodide concentration. There is, however, a maximum limit of inhibition by the protein at any given iodide concentration. The apparent competition between the two substrates, protein and iodide, without ever achieving complete inhibition, is consistent with two separate sites on the enzyme for the substrates.

Furthermore, the kinetic properties of the iodination reaction are also consistent with a two-site model. Measurement of the initial rate is somewhat complicated by the presence of a time lag, but this difficulty is essentially eliminated at higher concentrations of iodide (Fig. 3) although the concentration must be kept sufficiently low to avoid, as we will show later, inhibition of the iodination of protein by I_2 formation (Fig. 4). The steady-state velocity of the iodination reaction exhibits a sigmoid relationship with respect to iodide concentration which becomes progressively more accentuated with increasing protein concentration (Fig. 4). The sigmoidicity cannot in this case be attributed to a second-order bimolecular reaction of the type associated with I_2 formation (Fig. 1). On the other hand, a model based on the two-site hypothesis with the added assumption of a random order of attachment of the two substrates (Fig. 5) leads to a steady-state equation in terms of substrate concentrations and rate constant which is fully consistent with the sigmoid relationship. The following simplified steady-state equation for the model was derived by the method of King and Altman [10]:

$$\frac{V}{M} = \frac{A\,B\,(a + b\,A + c\,B)}{A\,B\,(a + b\,A + c\,B) + d\,A\,B + e\,A + f\,A^2 + g\,B + h\,B^2 + i}$$

where V = steady-state velocity of iodination of protein, A = iodide concentration, B = protein concentration, k_1 to k_9 = rate constants as indicated in Fig. 5 and M = a constant equal to $k_5 E_t$ in which E_t equals the total enzyme content; a, b, c, d, e, f, g, h and i are the following products of the various rate constants:

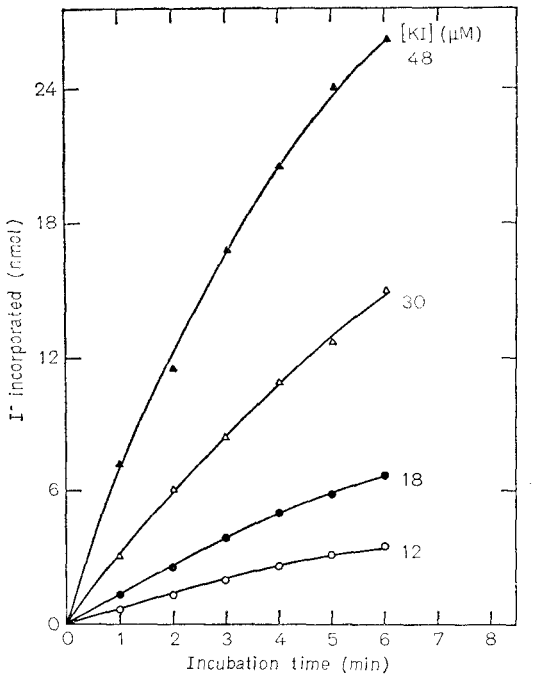

Fig. 3. *Time course of peroxidase-catalyzed iodination of lysozyme at pH 4.8 and at various concentrations of iodide.* The reaction mixtures contained 0.05 M acetate buffer pH 4.8, 1.48 mM lysozyme, 5 mM glucose, 7 nm glucose oxidase, 25 nM horse-radish peroxidase and potassium iodide concentrations as indicated. The reactions were carried out at 37 °C. (▲) 48 μM KI, (△) 30 μM KI, (●) 18 μM KI and (○) 12 μM KI

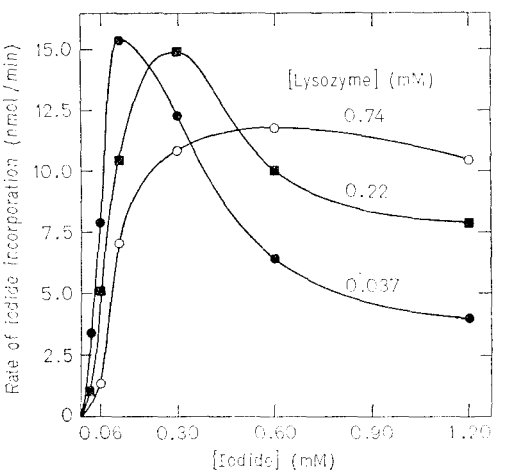

Fig. 4. *Influences of iodide and lysozyme concentrations on the initial rate of the peroxidase-catalyzed iodination of lysozyme at pH 4.8.* The contents of the reaction mixtures were the same as those in Fig. 3, except that the peroxidase concentration was 10 nmM and the lysozyme and iodide concentrations were varied as indicated in the figure. The incubations were carried out at 37 °C. (○) 0.74 mM, (■) 0.22 mM and (●) 0.037 mM lysozyme

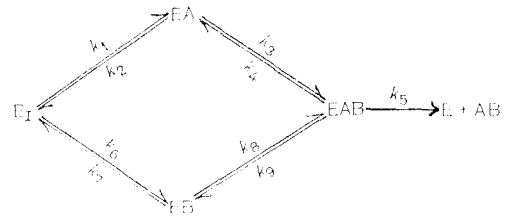

Fig. 5. *Reaction scheme for peroxidase-catalyzed iodination of lysozyme based on 2-site model with random order of substrate addition.* E_I represents horse-radish-peroxidase · H_2O_2 complex; A and B represent iodide and lysozyme, respectively

$$a = k_1k_3k_7 + k_2k_6k_8$$
$$b = k_1k_3k_8$$
$$c = k_3k_6k_8$$
$$d = k_3k_5k_8 + k_4k_6k_8 + k_1k_3k_9$$
$$e = k_2k_4k_8 + k_2k_5k_8 + k_1k_4k_7 + k_1k_7k_9 + k_1k_5k_7$$
$$f = k_1k_4k_8 + k_1k_5k_8$$
$$g = k_3k_7k_9 + k_3k_5k_7 + k_2k_6k_9 + k_2k_4k_6 + k_2k_5k_6$$
$$h = k_3k_6k_9 + k_3k_5k_6$$
$$i = k_2k_4k_7 + k_2k_5k_7 + k_2k_7k_9$$

For a constant protein concentration (B), this equation is simplified further to the form:

$$\frac{V}{M} = \frac{\alpha A^2 + \beta A}{\gamma A^2 + A + \delta}.$$

It can be seen that if α is sufficiently greater than β, γ is sufficiently small and δ sufficiently large, then V/M may approach $\alpha A^2/\delta$ with decreasing iodide concentration, A. At low iodide concentrations (A), therefore, the relationship between V and A would be second order. As A progressively increases, the relationship between V and A gradually changes from second order to first order, and eventually to zero order. Because of the B and B^2-containing terms in the denominator, incorporated particularly in δ, the second-order relationship is favored by greater protein concentrations, B. This is precisely the situation observed with the peroxidase-catalyzed iodination of lysozyme, the higher the protein concentration, B, the more accentuated the sigmoidicity of the relationship between V and iodide concentration. In Fig. 6 is illustrated the experimental relationship observed between the steady-state rate of lysozyme iodination and the iodide concentration at pH 4.8 and over a low concentration range of iodide, conditions under which I_2 formation is negligible. The sigmoidicity of the relationship is apparent and curve-fitting by means of the MLAB program [9] and a PDP-10 computer confirmed that the sigmoid relationship was a far better fit to the data over this low range of iodide concentrations than a straight line. These experimental results are, therefore, consistent

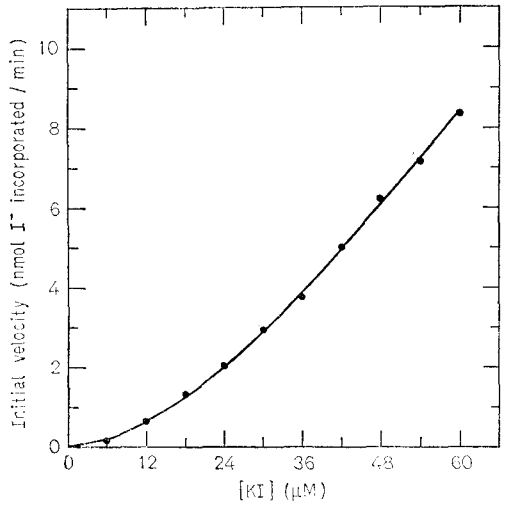

Fig. 6. *Influence of iodide concentration on initial rate of the peroxidase-catalyzed iodination of lysozyme.* The incubation conditions were the same as those in Fig. 3, except that the iodide concentration was varied as indicated. Each point represents the mean of the values obtained in six experiments. The solid line represents the theoretically computed best-fitting curve of all the data to the rate equation derived from the two-site, random-sequence model for peroxidase-catalyzed protein iodination (see text). (●) Experimental data and (——) theoretical fit for two-site model

with the model described above. However sigmoidicity is not necessarily a general property of the model and its presence is dependent on appropriate relative values of the individual rate constants. The evaluation of the individual rate constants in the peroxidase-catalyzed iodination of lysozyme or even a comprehensive analysis of the constraints which are necessary for sigmoidicity to appear have not been undertaken. The latter has been accomplished by Ferdinand [11], who has proposed the same model as a possible kinetic basis for sigmoidicity under some conditions. One minimum condition for sigmoidicity is apparent; although the model is for a bimolecular reaction with a random sequence of substrate addition, sigmoidicity appears only when one pathway to the ternary complex is kinetically preferred.

This mechanism can account for many of the properties of the peroxidase-catalyzed protein-halogenation reaction. Sigmoidicity with respect to iodide concentration would results if the following conditions are met: (a) the free enzyme has a greater affinity for the lysozyme than for iodide, (b) the addition of the iodide to the enzyme is inhibited by the presence of protein on the enzyme and (c) the addition of lysozyme to the enzyme is not seriously impaired by the presence of iodide on the enzyme. Low iodide concentrations would then favor the E → EB → EAB pathway where E = enzyme, B = protein and A = iodide, but increasing iodide

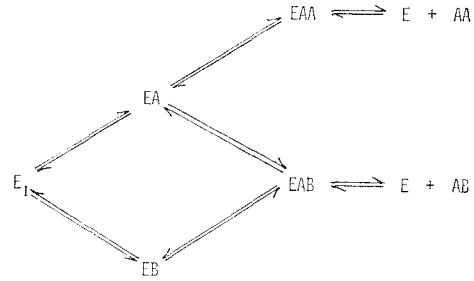

Fig. 7. *Model for the alternative pathways leading to either I_2 formation or iodinated protein, depending on the ratio of the concentrations of the iodide and protein substrates.* E_I represents peroxidase · H_2O_2 complex (complex I); A and B represent iodide and lysozyme, respectively

concentrations would progressively shift the reaction to the overall kinetically preferred pathway E → EA → EAB, thus resulting in sigmoidicity.

Influence of pH on the Nature of the Product Formed

If one examines the rate of iodination of lysozyme as a function of iodide concentration, an inhibition by higher iodide concentrations is observed which becomes progressively greater with decreasing lysozyme concentration (Fig. 4). I_2 is being produced when the inhibition by excess I^- of protein iodination begins to be observed. When the iodide/protein concentrations ratio rises, I_2 formation increases and protein iodination decreases. Fig. 7 offers a model which accounts for both reactions, I_2 formation and protein iodination.

Further experiments have shown that I_2 formation is favored at lower pH. When the reaction is studied at higher pH, *e.g.* pH 6,0, significant inhibition of lysozyme iodination by excess iodide is not observed over the same range of iodide concentrations up to 1.2 mM (Fig. 8). Some I_2 may be formed under these conditions and the rate of lysozyme iodination may, perhaps, level off more sharply than normally excepted, but the rate of protein iodination does not decline. Inhibition by excess iodide, therefore, varies with pH. Fig. 9 illustrates the influence of pH on the rate of lysozyme iodination at two widely different iodide concentrations and a single concentration of lysozyme. The inhibition by excess iodide is seen at low pH and when the ratio of protein to iodide concentration is low. That the inhibition is associated with I_2 formation is evidenced by the direct measurement of I_2 formation as a function of pH both in the absence and presence of lysozyme (Fig. 10). I_2 is the only product of the reaction at pH values up to about 4. Between pH 4 and 6 the halogenation of protein becomes progressively favored and I_2 formation inhibited. Conversely, inhibition

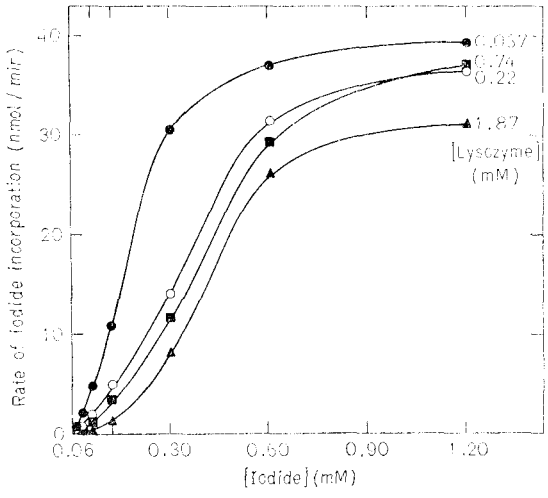

Fig. 8. *Influence of iodide and lysozyme concentrations on the initial rate of the peroxidase-catalyzed iodination of lysozyme at pH 6.0.* The reaction mixtures contained 0.05 M phosphate buffer pH 6.0, 66 nM glucose oxidase, 0.25 μM horse-radish peroxidase and various concentrations of lysozyme and KI as indicated in the figure. Incubations were carried out at 37 °C. (●) 0.037 mM, (○) 0.22 mM, (■) 0.74 mM and (▲) 1.87 mM lysozyme

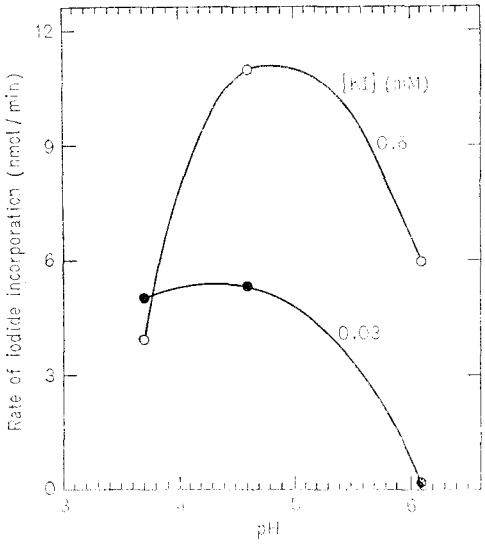

Fig. 9. *Influence of pH on initial rate of peroxidase-catalyzed iodination of lysozyme.* The reaction conditions were the same as those in Fig. 4, except that the concentration of lysozyme was 0.375 mM and the pH and KI concentration were varied as indicated: (○) 0.6 mM KI and (●) 0.03 mM KI

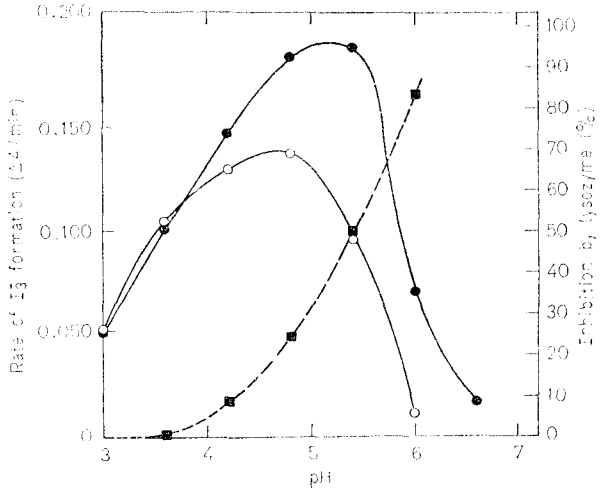

Fig. 10. *Influence of pH on the lysozyme inhibition of the peroxidase-catalyzed oxidation of I^- to I_2.* I_2 formation was determined by measurement of absorbance at 353 nm (see Methods). The rates represent initial velocities. The reaction conditions were similar to those described in Fig. 4, except that the KI concentration was 1.2 mM, the lysozyme concentration, when present, was 0.75 mM, the horse-radish peroxidase concentration was 25 nM, and the buffer was 0.05 M citrate varying in pH as indicated. Incubation temperature was 24 °C. The percentage inhibition by lysozyme (■——■) was calculated on the basis of the difference in the initial rates of I_2 formation in the presence (○——○) and absence (●——●) of lysozyme in the reaction mixture, measured by change in absorbance per min

of the protein iodination reaction at pH 6—6.5 by excess I^- is very slight and much less significant than at more acidic pH.

These results suggest that the affinity of the sites on the enzyme for iodide is greater at more acidic pH; at more neutral pH one of the sites retains its affinity for iodide while in the other site, affinity for iodide decreases as the affinity for the protein, presumably a tyrosine residue in the protein chain, rises. It might be expected then that at more alkaline pH, the formation of tyrosine dimers from free tyrosine might be favored, both sites on the enzyme exhibiting a much higher affinity for tyrosine than for iodide. Indeed such is the case (Fig. 11). The model proposed in Fig. 7 may therefore be extended (Fig. 12).

Some Quantitative Data on the Nature of the Product Formed when the Relative Concentrations of I and Enzyme Are Varied

Roman and Dunford [8] have titrated complex I with I^- using a spectrophotometric technique. Complex I was prepared at pH 8.4 using 2.7 μM peroxidase and 2.2—8.8 μM H_2O_2. They assumed that under these conditions all the H_2O_2 present in complex I was used to oxidize I^-, *i.e.* that no enzyme iodination occurred. This assumption was based on results obtained by measuring the stoichiometry of the reaction:

$$H_2O_2 + 2\,I^- + 2\,H^+ \xrightarrow{\text{Peroxidase}} I_2 + 2\,H_2O$$

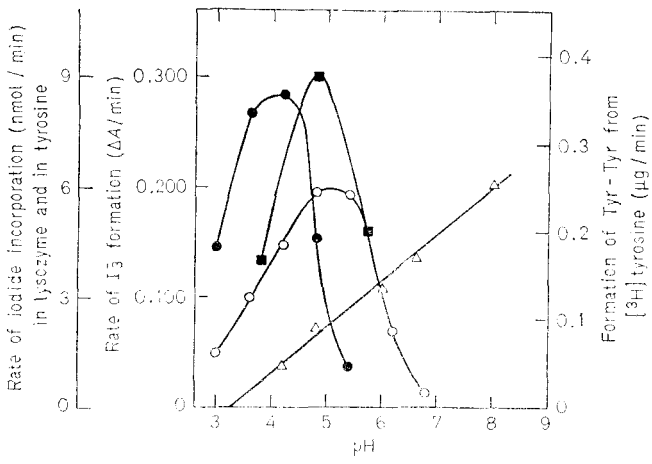

Fig. 11. *Influence of pH on the rates of each of the following reactions catalyzed by horse-radish peroxidase: (a) iodination of lysozyme (■——■) (b) iodide oxidation and I_2 formation (○——○), (c) iodination of free tyrosine (●——●) and (d) tyrosine oxidation and dimerization (△——△).* In all cases the reaction mixtures contained in a final volume of 1.0 ml: 5 mM glucose, 66 μM glucose oxidase and 0.05 M sodium acetate or phosphate buffer varying in pH as indicated. In addition, the specific conditions in the assay of each of the reactions were as follows: (a) 0.375 mM lysozyme 0.6 mM KI, 0.5 μM horse-radish peroxidase and an incubation temperature of 37 °C; (b) 1.2 mM KI, 0.05 μM peroxidase and incubation temperature of 24 °C; (c) 0.11 mM tyrosine, 0.93 mM KI, 0.025 μM peroxidase and incubation temperature of 37 °C; (d) 0.27 mM tyrosine, 0.05 μM peroxidase and incubation temperature of 37 °C. The assay of free tyrosine oxidation and dimerization was subject to some imprecision because of the relative insolubility of the product

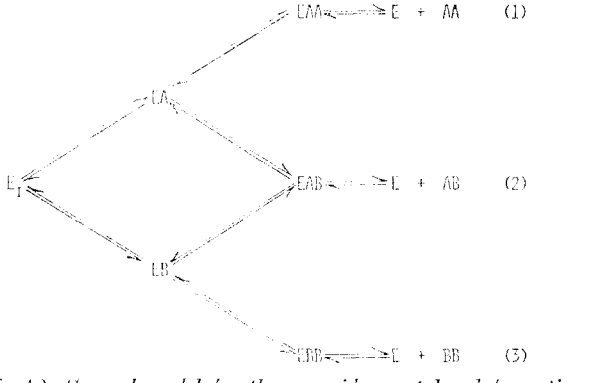

Fig. 12. *General model for the peroxidase-catalyzed formation of I_2, iodinated protein or tyrosine dimer.* Pathway (1) is favored at acidic pH, pathway (3) at alkaline pH and pathway (2) at intermediate pH. A represents iodide, B represents protein or free tyrosine and E_I represents peroxidase · H_2O_2 complex (complex I)

This stoichiometry was verified at a lower pH (7.4) and with different concentrations of peroxidase (4.6 μM) and iodide (10 mM). From our results presented above we suspected that since the ratio

Table 1. *Relationship between H_2O_2 utilization and I_2 formation or protein iodination at high enzyme concentration*
The horse-radish-peroxide-catalysed oxidation of I^- to I_2 and the self iodination of the peroxidase were compared at pH 7.24 and 8.4. The buffer was 0.05 M phosphate. The other components of the reaction mixtures are indicated in the Table. The results are expressed as the percentage H_2O_2 used in 30 s either to produce I_2 or to iodinate the enzyme

pH	Concentration of			H_2O_2 used for	
	H_2O_2	Peroxidase	I^-	I_2 formation	Peroxidase iodination
	μM	mM	mM	%	%
7.24	10	0.0044	10	100	0
7.24	10	0.0027	0.25	15 20	16
8.4	10	44.00	10	80	—
8.4	10	0.27	0.25	0	12

of I^-/peroxidase concentrations was 25-fold lower in the titration experiment, some iodination of the enzyme could occur. Table 1 shows that this is indeed the case. The titration experiment performed by Roman and Dunford [8] was, therefore, not suitable to measure the stoichiometry between H_2O_2 utilization and I_2 formation. These experiments confirm that the nature of the product depends greatly on the relative concentrations of the iodide and enzyme. When present at high concentration, the enzyme behaves as a protein substrate.

Free Tyrosine Iodination

Horse-radish peroxidase is reputed to be unable to iodinate free tyrosine [12]. The results described above could explain why this reaction might not be obtained at pH values near neutrality, where the affinity for the phenolic acid favors tyrosine-tyrosine formation, or in very acidic media, where I_2 formation occurs most readily. Under proper conditions at an acid pH low enough to prevent significant tyrosine-tyrosine formation, it has been possible to obtain peroxidase-catalyzed iodination of free tyrosine (Fig. 13). Both monoiodotyrosine and small amounts of diiodotyrosine are obtained. An additional iodinated product remains at the origin on paper chromatographic analysis, probably, the dimer of monoiodotyrosine. With increasing pH, tyrosine dimer formation is increased which reduces the rate of halogenation of the free tyrosine. The pH optimum for iodination of the free amino acid is, therefore, lower than that for the iodination of the same species of amino acid contained within lysozyme. This discrepancy probably reflects the increased competition for tyrosine dimer formation which occurs with increasing pH in the case of the free amino acid; this reaction is unlikely to occur with the protein because of the improbability of two

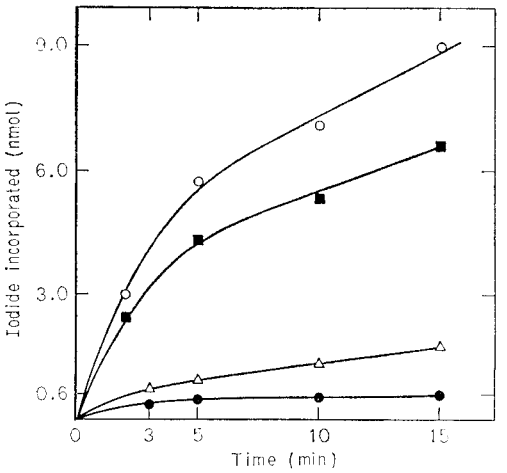

Fig. 13. *Time course of peroxidase-catalyzed iodination of free tyrosine.* The products were determined by paper chromatographic separation (solvent: ethanol–0.2 M ammonium carbonate, 2:1, v/v) and assay of ^{125}I radioactivity in an Autogamma scintillation spectrometer. The reaction mixture contained 1 mM glucose, 0.066 μM glucose oxidase, 0.11 mM tyrosine, 0.05 M sodium acetate buffer pH 4.8, 0.25 μM horse-radish peroxidase and 0.93 mM KI containing 2 μCi $^{125}I^-$ in a final volume of 1.0 ml. Incubation temperature was 37 °C. (○) Total organic iodide, (■) monoiodotyrosine, (△) origin material and (●) diiodotyrosine

molecules of receptor protein being situated on the enzyme at the same time. However tyrosine-tyrosine bridges have been shown to occur in some proteins [13, 14].

Analogous results are obtained when monoiodotyrosine is the substrate. The product is diiodotyrosine, and the optimum pH is identical to that for the monoiodotyrosine formation. The observation that free tyrosine can be iodinated by peroxidase with proper conditions of pH is consistent with the two-site hypothesis and with the conclusion that the nature of the product can be modified by changing the relative affinity of each one of these two sites for its substrates.

DISCUSSION

Several mechanisms are theoretically capable of explaining the formation of I_2 from I^-, catalyzed by horse-radish peroxidase. Two would predict first-order kinetics with respect to iodide concentration.

$$\text{Model (1): } E_I + 2I^- \leftrightarrows E + 2I^\circ \quad 2I^\circ \leftrightarrows I_2$$

$$\text{Model (2): } E_I + I^- \leftrightarrows E + I^+ \quad I^+ + I^- \leftrightarrows I_2.$$

The peroxidase · H_2O_2 complex, E_I (complex I), contains two equivalents of oxidation. It can, therefore, remove one electron from each of two molecules of substrate (I^-), I° being produced (Model [1]); two I° atoms are released in the medium where they dimerize to I_2. According to Model (2), E_I removes two electrons from a single I^- molecule, producing free I^+ which leads to I_2 by dismutation with I^- present in excess in the medium. According to these two mechanisms, I_2 is formed from either I° or I^+, previously released into the medium. These two mechanisms would not lead to second-order kinetics. Models (3) and (4) are, however, consistent with a bimolecular reaction between one mole of enzyme and two moles of substrate, the product being released only after dimerization or dismutation of the oxidized species on the surface of the enzyme.

$$\text{Model (3): } E_I + 2I^- \leftrightarrows E\,(2I^\circ) \leftrightarrows E\,(I_2) \rightleftharpoons E + I_2$$

$$\text{Model (4): } E_I + 2I^- \leftrightarrows E\,(I^+)\,(I^-) \rightarrow E\,(I_2) \rightarrow E + I_2$$

In our experiments with the peroxidase, the experimental results at pH 6.9 fit a second-order reaction; however, at more acidic pH, the degree of sigmoidicity is reduced suggesting that the reaction could also be partly first order. Recently, Maguire and Dunford [15] have found with lactoperoxidase both a first-order and a second-order dependence on the concentration of iodide ion.

The choice between Models (3) and (4) (*i.e.* which is the oxidized species, I^+ or I°) appears to be much more difficult. Data obtained with myelo- and thyroid peroxidase by Yip and Hadley [16] and with horse-radish peroxidase by Nunez and Pommier [1,3] and Bjorkstein [17], suggest indirectly that I° is actually produced. However, Roman and Dunford [8] concluded on the basis of the results of spectrophotometric titration of the peroxidase complex I with I^- that one mole of complex I (1 mole H_2O_2/mole peroxidase) oxidizes only one mole iodide. This result would be consistent with the equation,

$$\text{Peroxidase} \cdot H_2O_2 + I^- \rightarrow \text{Peroxidase} + I^+$$

the I^+ not necessarily being released into the medium. This conclusion would be valid if no iodination of the enzyme occurred. Using the experimental conditions of Dunford [8], we have found evidence of iodination of the peroxidase. Moreover, we have verified that at the pH used by these authors no I_2 is produced. Roman and Dunford [8] believed that they excluded iodination of peroxidase by establishing the stoichiometry of the reaction,

$$H_2O_2 + 2I^- + 2H^+ \xrightarrow{\text{Peroxidase}} I_2 + 2\,H_2O_2$$

The conditions used for the titration and stoichiometry experiments were very different, however, since the pH was higher in the titration experiment as well as the enzyme/iodide ratio (25 times higher). The conclusions of the titration experiment devised

by Roman and Dunford [8] are, therefore, not valid because at high concentration, horse-radish peroxidase behaves not only as an enzyme but also as a protein substrate.

Moreover, our results show that: (a) the higher the pH the higher the yield of protein iodination and the lower the I_2 formation and (b) the lower the iodide/protein acceptor ratio, the lower the I_2 yield.

In other words, the proportions of the two products, I_2 and iodinated protein, depend greatly on the relative concentrations of the two substrates.

It seems, therefore, very difficult to chose on the basis of kinetic and titration experiments between Models (3) and (4). The only point which seems clearly established is that the reaction of I_2 formation is at least partly second order with respect to iodide concentration, suggesting again [2] the presence of two sites for the substrate at the surface of the peroxidase. Our kinetic studies on protein iodination catalyzed by the peroxidase agree with this conclusion.

According to this model one site binds the iodide and the second one the protein. It is, therefore, very likely that the protein (*i.e.* its tyrosine residues) is a substrate in the reaction being oxidized by peroxidase according to Model (5):

Model (5): $E_I + I^- + \text{Tyr-Protein} \rightleftarrows E\begin{matrix}\diagup I^\circ \\ \diagdown \text{Tyr}^\circ\text{-Protein}\end{matrix} \rightarrow E + \text{Protein-I.}$

However, as in the case of I_2 formation, Model (6) cannot be excluded:

Model (6): $E_I + I^- + \text{Tyr-Protein} \rightleftarrows E\begin{matrix}\diagup I^+ \\ \diagdown \text{Tyr-Protein}\end{matrix} \rightarrow E + \text{Protein-I.}$

It is difficult to establish a direct proof that the tyrosine residues are oxidized during the protein iodination reaction. That free tyrosine or protein tyrosine are good substrates for peroxidase is, however, strongly suggested by various data. Gross and Sizer [18] showed many years ago that the ultraviolet spectrum of different proteins is modified in the 280-nm region after incubation with peroxidase and that their enzymatic activity is suppressed. They showed also that oxidation of free tyrosine by peroxidase yields radicals of this amino acid which polymerize in solution. Nunez and Pommier [2] have also obtained data suggesting that tyrosine residues of the protein substrate are oxidized: for instance, the protein is a competitive inhibitor of guaiacol oxidation *etc.* It is therefore likely that tyrosine residues are oxidized to radicals by peroxidase. However, it is very difficult to devise unequivocal experiments to prove this directly when the protein or free tyrosine are involved in the iodination reaction. Therefore, the minimal conclusions from this work are that the protein interacts with the enzyme and can compete with iodide; this interaction is pH dependent; the formation of the various products of the reaction (I_2, iodinated protein, free iodotyrosine, tyrosine dimer) is favored differently at different pH (Fig. 13). These observations are consistent with two sites for substrate binding on the peroxidase molecule. Moreover, these sites must be relatively non-specific and able to bind either two molecules of the same substrate or two different ones.

Another likely conclusion is that I_2 cannot be the iodinating species and Models (7) or (8) cannot apply.

Model (7): $E_I + 2I \rightleftarrows E + I_2$
and $I_2 + \text{Tyr} \rightarrow \text{Monoiodotyrosine}$

Model (8): $E_I + I^- \rightleftarrows E + I^+$;
$I^+ + I^- \rightleftarrows I_2$ and $I_2 + \text{Tyr} \rightarrow \text{Monoiodotyrosine}$

As we [2] for horse-radish peroxidase and Taurog [19] for thyroid peroxidase have already pointed out, the protein iodination reaction is always inhibited when formation of I_2 is observed. Moreover, the pH optimum for the protein iodination reaction catalyzed by peroxidase is equal to 4.8. At this pH the yield of iodination of tyrosine by I_2 is very low since it depends [20] on alkaline catalysis both of phenoxide formation and of deprotonation in the 3′ (or 5′) position(s). For these reasons Models (7) and (8) appear unlikely.

The authors wish to express their gratitude to Mme de Prailauné for her excellent technical assistance and to Dr Clifford Patlack of the Theoretical Statistics and Mathematics Branch, National Institute of Mental Health, for his advice on mathematical matters and the performance of the computer analyses.

REFERENCES

1. Nunez, J. & Pommier, J. (1968) *Eur. J. Biochem. 5* 114–118.
2. Nunez, J. & Pommier, J. (1969) *Eur. J. Biochem. 7*, 286–293.
3. Alexander, N. M. (1962) *Anal. Biochem. 4*, 341–345.
4. Morrison, M., Bayse, G. S. & Michaels, A. W. (1971) *Anal. Biochem. 42*, 195–201.
5. Mann, P. J. (1931) *Biochem. J. 25*, 918–930.
6. Chance, B. (1949) *Arch. Biochem. 21*, 416–430.
7. Weinryb, I. (1966) *Biochemistry, 5*, 2003–2008.
8. Roman, R. & Dunford, H. B. (1972) *Biochemistry, 11*, 2076–2081.
9. Knott, G. D. & Reece, D. K. *Proceedings at the Online '72 International Conference*, Brunel University, England, Sept., 1972.
10. King, E. L. & Altman, C. (1956) *J. Phys. Chem. 60*, 1375–1381.

11. Ferdinand, W. (1966) *Biochem. J. 98*, 278–283.
12. Ljunggren, J. G. (1966) *Biochim. Biophys. Acta*, *113*, 71–78.
13. LaBella, F., Waykole, P. & Queen, G. (1968) *Biochem. Biophys. Res. Commun. 30*, 333–338.
14. Keeley, F. W., LaBella, F. & Queen, G. (1969) *Biochem. Biophys. Res. Commun. 34*, 156–163.
15. Maguire, R. J. & Dunford, H. B. (1972) *Biochemistry*, *11*, 937–941.
16. Yip, C. C. & Hadley, L. D. (1966) *Biochim. Biophys. Acta*, *122*, 406–412.
17. Bjorkstein, F. (1970) *Biochim. Biophys. Acta*, *212*, 407–416.
18. Gross, A. J. & Sizer, I. W. (1959) *J. Biol. Chem. 234*, 1611–1614.
19. Taurog, A. (1970) *Arch. Biochem. Biophys. 139*, 212–220.
20. Mayberry, W. E., Rall, J. E. & Bertoli, D. (1964) *J. Am. Chem. Soc. 86*, 5302–5307.

J. Pommier, L. Sokoloff, and J. Nunez, Unité de Recherche sur la Glande Thyroïde et la Régulation Hormonale. I.N.S.E.R.M., 78 Avenue du Général-Leclerc, F-94270 Le Kremlin Bicêtre, France